AWARD NUMBER: W81XWH-15-1-0631

TITLE: Dissecting Neuronal Participation to Focal Epileptic Events in Vivo

PRINCIPAL INVESTIGATOR: Stelios M Smirnakis MD PhD

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston TX 77030

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sep 2015 - 29 Sep 2016

4. TITLE AND SUBTITLE

Dissecting Neuronal Participation to Focal Epileptic Events in Vivo

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0631

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Stelios M Smirnakis MD PhD

E-Mail: smsmirnakis@partners.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine,
1 Baylor Plaza
Houston
TX, 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Epilepsy is prevalent among Veterans conferring significant morbidity. Despite decades of study, its circuit mechanisms remain poorly understood. To develop rational approaches to therapy we need to understand the role that individual neurons of different types play in epileptic events. The two-photon, "optical micro-encephalogram" is a powerful tool for dissecting circuit mechanisms of epilepsy, by allowing us to follow the activity of individual units chronically in vivo. We use this method and patch-clamp to study the well-validated Tetanus Toxin model of focal epilepsy. Our goal is to map how different types of cortical neurons get recruited to seizures and epileptiform EEG events over time as focal epilepsy sets in, while identifying the role that different cell types play in its manifestations. In this 1st report, from 10/1/15 to 6/1/2016, we met our SOW goals to hire/train a dedicated postdoc, develop the experimental paradigm, and begin studying the role of pyramidal neurons. We request the transfer of the award to Boston JP VA, where the PI relocated, without additional cost. Aims and key personnel will remain identical.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	14	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION

The high prevalence of epilepsy among veteran populations with traumatic brain injury (TBI) makes epilepsy one of the congressionally directed topic areas. In previous studies, the electroencephalography (EEG) recording at the cortical surface during TBI-induced epileptic seizures revealed hyper-synchronous epileptic bursts, whereas single-cell recordings found heterogeneous neuronal spikes during the hyper-synchronous EEG bursts (Truccolo et al., 2011). To define the correlation between the EEG and single-neuron activity and to determine how different cell types participate in seizure events, we monitor the individual activity of a large number of neurons in vivo using 2-photon microscopy. As a model of the long-term effects of TBI, we inject tetanus toxin (TeT) into the visual cortex of mice to induce seizures. The activity-dependent calcium indicator GCamp6, which in our case is expressed in selective neurons by gene modification, or in all neurons by virus infection, reports the activity of individual neurons. Several types of neurons from multiple layers of the visual cortex are recorded at several time points. The experimental timeline is shown in Figure 1.

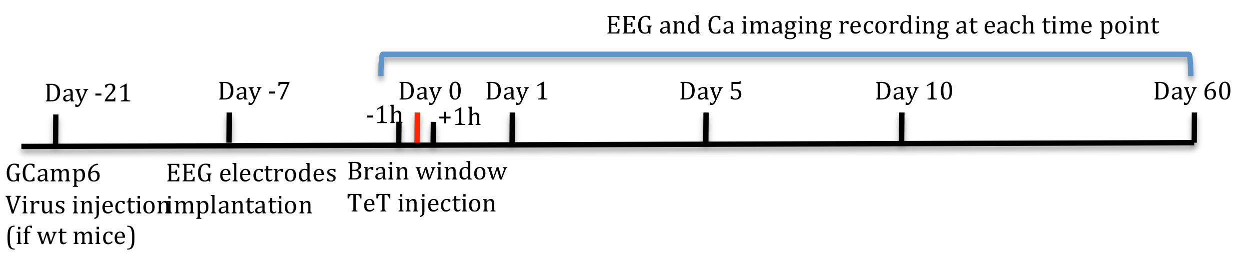

Figure 1 The experimental time line.

2. KEYWORDS:

synchronization,
excitation and inhibition balance,
systems neuroscience,
visual cortex,
traumatic brain injury,
epilepsy,
tetanus toxin,
seizure,
GCamp6
calcium indicators,
patch-Clamping

3. ACCOMPLISHMENTS

Major Goals and Objectives:
(as stated in the original SOW, site: Baylor College of Medicine)

START DATE OF THE AWARD: October, 1, 2015
TRANSFER DATE OF THE AWARD : June 1, 2016.
PERIOD COVERED BY THIS REPORT: 10/1/2015 – 6/1/2016

Specific Aims 1, 2 will proceed in parallel	Timeline	Site 1
Study pyramidal and PV+ interneuron cohorts	Months	BCM (Smirnakis)
Hire a new postdoc, train personnel, Set up the TeT injection experiments, IACUC approval	1-4	BCM
Have SA#1,2 proceed in parallel, studying the pyramidal neurons.	4-16	BCM
Studying PV+ interneuron cohorts.	9-24	BCM
Milestone(s) To Achieve:		
Local IRB/IACUC Approval	1-3	BCM
Write a first manuscript.	16-24	BCM
Study SOM+ and VIP+ interneuron cohorts		
Have SA#1,2 proceed in parallel studying SOM+ interneurons	16-30	*BCM*
Have SA#1,2 proceed in parallel studying VIP+ interneurons	24-34	*BCM*
Write 1-2 additional manuscripts	30-36	*BCM*
Milestone(s) Achieved: 1. 1-2 Manuscripts	30-36	*BCM*

What Was Accomplished (for the 9 month period covered by the grant) :

Months 1-4: All goals described in the SOW for months 1-4 were met. Specifically,

1) *A new postdoctoral fellow, 100% dedicated to this project was identified and hired*: We identified and hired Dr. Zhaozhe Hao, a Cellular and Behavioral Neurobiology Ph.D., whose time is dedicated 100% to this project.
2) Dr Jochen Meyer a senior postdoctoral fellow promoted to Instructor in the Department of Neurology at Baylor College of Medicine, also worked on the DoD project, dedicating 30% of his time to this effort.
3) *Personnel was trained in the procedures required*: Dr Smirnakis originally trained Dr Meyer. Dr. Hao took all the required training from Baylor College of Medicine. Dr. Jochen Meyer, an Instructor In the dept. of Neurology, and Dr Smirnakis trained Dr. Hao in all experimental techniques. Dr. Hao has been able to conduct experiments independently following the period of training.
4) IACUC approval for these experiments was obtained at Baylor College of Medicine.

5) *<u>TeT injection aexperiments to model chronic focal epilepsy were setup.</u>* The experimental setup combining the EEG recording, calcium imaging and mouse behavior monitoring was finished in March, 2016.

<u>Month 4-16 (</u>Use 2-photon and patch-clamp strategies in parallel to study pyramidal neuron activation during seizure events): This effort was initiated and data collection started as planned. However, the data collection was delayed as Dr Smirnakis laboratory prepared for the 6/1/2016 move to Boston (Brigham and Women's H. and JP VA H., Harvard Medical School). A further complication arose because of an error in handling Dr Hao's Visa by the Baylor Medical School International Office during transfer, which necessitated her return to China to reapply for a visa from there. This issue has since been resolved, and Dr Hao has returned at BVARI now to continue experiments.

These issues notwithstanding, significant progress has been made. Specifically:

1) The mouse colony of GCamp6s transgenic mice, whose pyramidal neurons fluoresce when they fire, was established. Specifically, we established two mouse colonies: C57/B6 wild type mice and a homozygous C57BL/6J-Tg(Thy1-GCaMP6s)GP4.3Dkim/J (GP4.3) colony that expresses the calcium-indicator Gcamp6 in pyramidal neurons. The wild type mice colony started from a breeding pair from Jackson lab. The colony was established for surgery practice and virus/toxin injection practice. The GP4.3 mice colony, which expresses the calcium-indicator Gcamp6 in pyramidal neurons was started from a heterozygous male ordered from Jackson lab. We confirmed that the F3 generation of homozygous GP4.3 mice have excellent GCamp6s expression when they reach 8 weeks of age (see Fig. 3c below). We observed no difference in appearance or behavioral between GP4.3 mice and the wild type mice. As of June 2016, we have produced 10 cages of good homozygous GP4.3 mice of both genders for experiments. The colony is in the process of being transferred to Boston to continue experiments.

2) <u>Established AAV-Gcamp6 virus injection procedure:</u> In order to be able to record from all neurons, including interneurons, in some experiments, we also established a virus injection procedure using AAV1.Syn.GCaMP6m.WPRE.SV40. The virus was injected at the imaging site in the mice visual cortex using a NamoJect injection pump, 3-4 weeks before the TeT injection (Figure 5).

3) <u>Established EEG recording procedure</u> : EEG recordings and 2-photon imaging experiments were started, and preliminary results obtained (see below): 1-2 weeks before the TeT injection, we implant the recording electrodes epidurally at 2.7mm lateral and 3-3.5 mm anterior to lambda on both hemispheres (Figure 2a). The reference electrode is implanted epidurally above the cerebellum contralateral to the TeT injection site. Each recording session consists of multiple segments of 20 minutes each, and the EEG is recorded at a sampling rate of 5000 Hz. 3/5 wild type mice were successfully implanted with EEG electrode and we recorded baseline activity. We also improved the mechanical stability of the EEG wire connector to prevent it from becoming detached over the 2 months after the implantation.

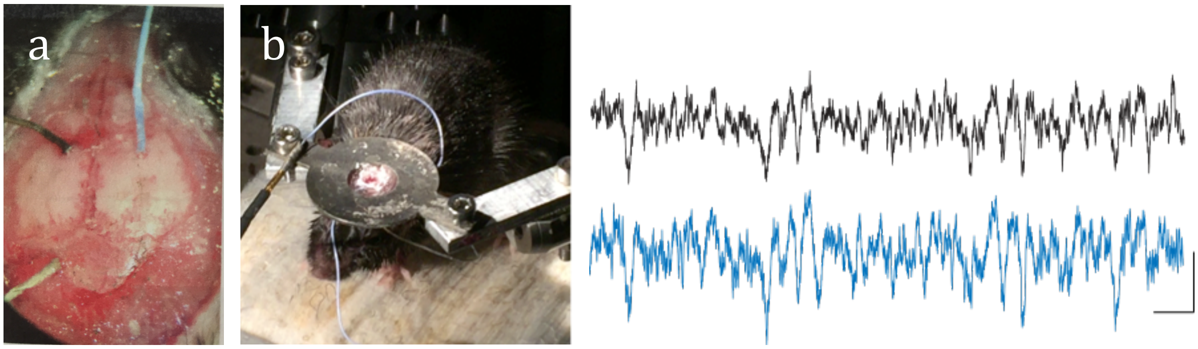

Figure 2. The experiment setup for EEG recordings.
a, the EEG recording electrodes were implanted bilaterally and rostral to the V1 cortex. The reference electrode was implanted above the cerebellum, contralateral to the side used for calcium imaging and TeT injection.
b, during EEG recordings, the mouse was stabilized with a headpost fixed on the skull. The mouse is allowed to run freely on a wheel.
c, a typical EEG recording before TeT injection. Simultaneous EEG recording from ipsilateral (upper trace) and contralateral (lower trace brain) locations. Upper trace, right side; lower trace, left side. Scale bar, 0.5 sec, 0.2 mV.

4) <u>Acquired refined surgical techniques to produce clear long-term cranial windows for calcium imaging and TeT injection.</u> The success rate for a working condition window immediately after surgery is about 70%. The success rate for cleaning and replacing old windows is about 50% (Figure 3).

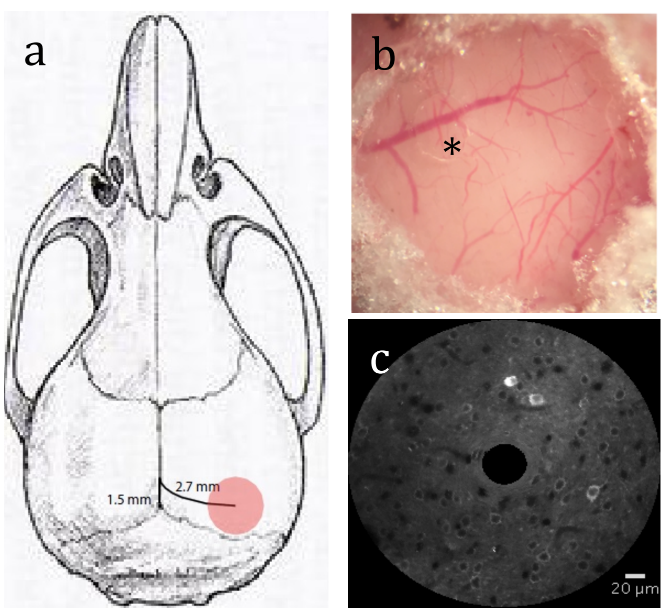

Figure 3. The craniotomy above the primary visual cortex enables Ca imaging and TeT injection.
a, a schematic drawing of a mouse skull showing the position of the window with a pink circle.
b, image of the window under a stereo microscope. *, hole in the glass coversliip for the entrance of the TeT injection pipette. Window diameter, 2mm.
c, GCamp6 labeled pyramidal neurons under the 2-photon microscope in a homozygous transgenic GP4.3 mouse.

5) <u>Combined and synchronized all components of the recording equipment.</u> The EEG recording, calcium imaging, and mouse behavior monitor were connected and an Matlab interface was programmed for synchronized data display (Figure 4).

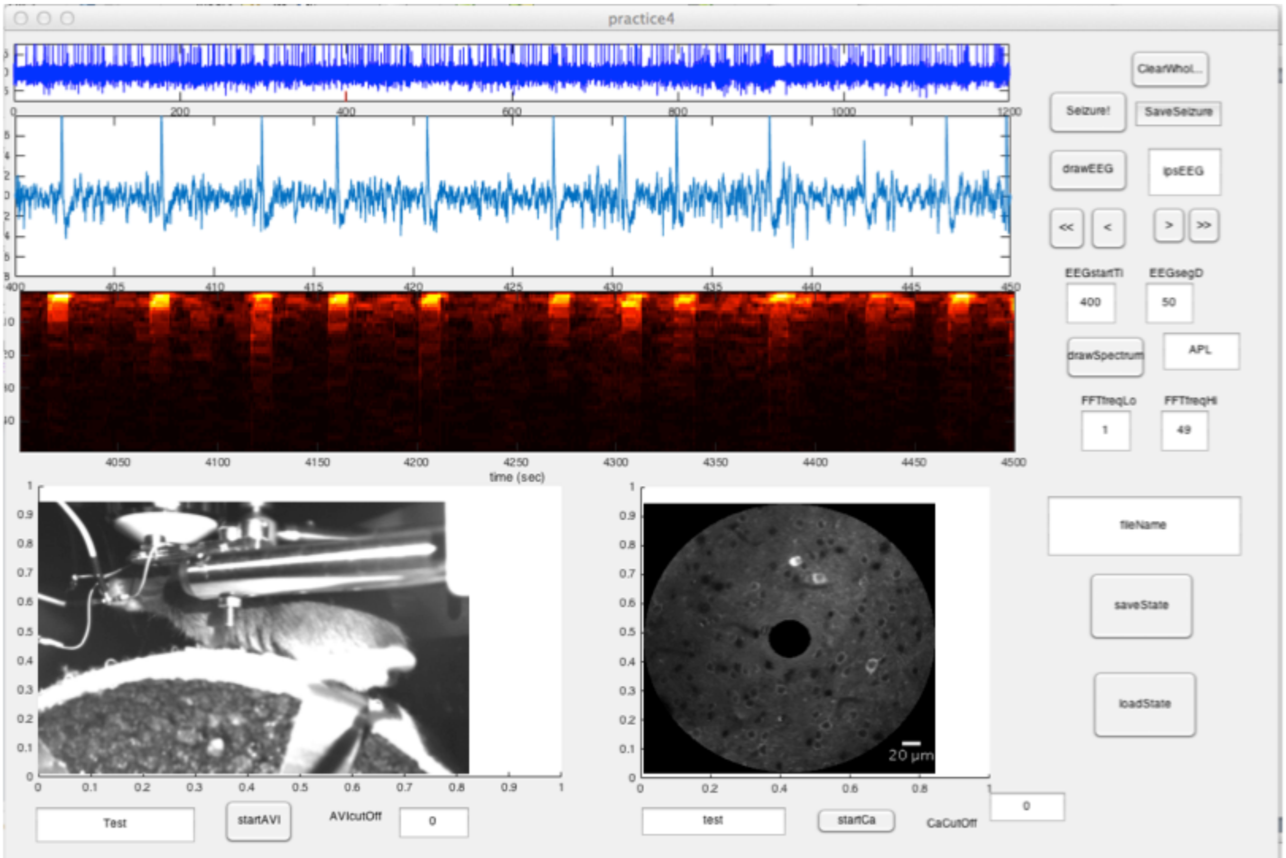

Figure 4. The Matlab interface is able to display the EEG trace over time, the EEG power spectrum, mouse behavior and the calcium imaging simultaneously. Top blue trace, 20 minutes of EEG recording. Second blue trace, a selected segment of the EEG recording showing above. Third panel on left, the power spectrum of the selected segment. Higher relative power is represented by brighter colors. Bottom left, an infrared video monitoring the mouse behavior during the EEG recording and calcium imaging (bottom right).

6) Established the procedure to inject TeT into primary visual cortex (V1).: We have successfully injected 7 mice with 0.2-2μL 0.75 ng/μL TeT. 3 of the injections were under visual guidance to confirm the exact injection location (Figure 5). Three of six mice displayed abnormal EEG activity and/or behavior immediately after the injection. 5 animals developed bilateral abnormal EEG activity. 5-10 days after the injection.

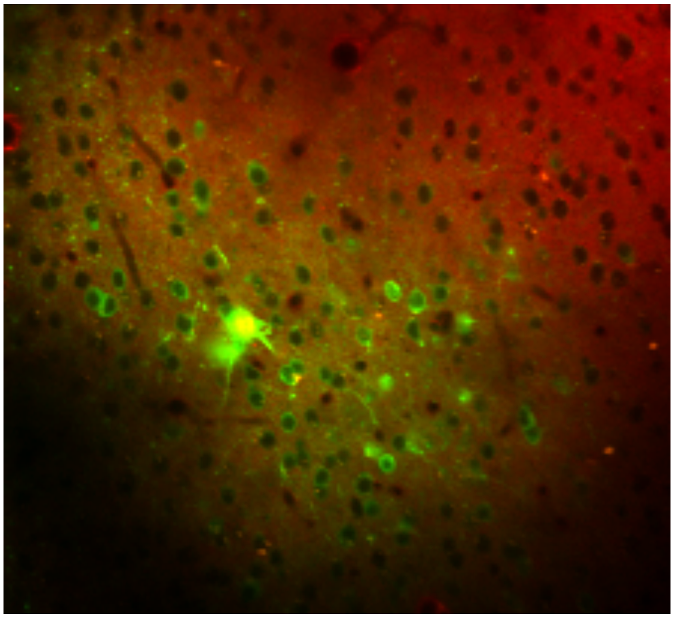

Figure 5. Green, GCamp6 loaded in all neuronal cell types by AAV-Gcamp6 virus injection 3 weeks before the TeT injection. 200 nLTeT solution were injected in cortical layer 5, and spread ~ 400 μm in radius to reache the calcium imaging area shown here in layer 2/3. Red, Alexa-495 dye contained in the TeT solution to visualize the injection. The image was taken at a depth of 150 μm. The injection was directly underneath the imaging site at a depth of 550μ.

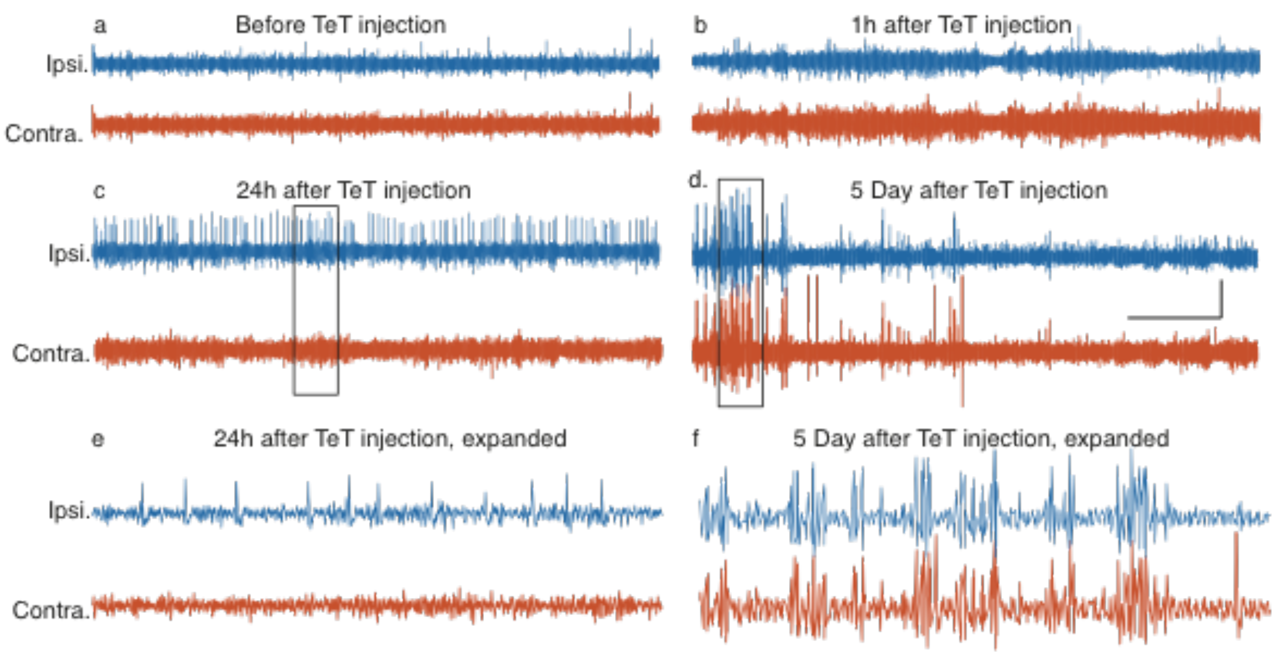

Figure 6. TeT injection into the primary visual cortex of mice induced long-term abnormal EEG activity. **(a-d):** EEG recordings >1mm away from the TeT injection site, before, and at 1 h, 24h and 5 days after TeT injections**.** Note the gradual development of rhythmic bursts of activity see non EEG. **(e-f):** Expanded EEG recordings from c and d. *Blue traces*: EEG recordings from the ipsilateral side of the TeT injection. *Orange Traces:* EEG recordings from the contralateral side of the TeT injection. Note the rhythmic epileptiform activity seen on EEG. Black box in c and d outlines the segments shown expanded in e and f. Scale bar, 1mV and 100 sec (a-d) or 8 sec (e and f).

We are currently continuing to perform 2-photon experiments with TeT injected animals in order to map how pyramidal neurons engage into seizure events. Dr Jochen Meyer is also

starting to perform patch clamp experiments to better delineate the temporal sequence of engagement of pyramidal neurons to EEG burst events across cortical layers. *We expect to adhere to the original timeline stated in the SOW (ie performing these experiments from 4-16 months following initiation of the project), after account is taken for the time that it will take to transfer the award to the new Institution. No new funds need to be requested for these unavoidable transfer delays.*

Opportunities for training and professional development:

i) Dr. Meyer trained Dr. Hao for mouse surgery, virus injection, EEG recording, Ca imaging and basic Matlab programming.

ii) Dr. Noebels and his trainee Dr Atul Maheshwari, our epileptology collaborators at Baylor College of Medicine, Dept. of Neurology, with several years of experience in human and mice EEG interpretation, trained Dr. Meyer and Dr. Hao in EEG reading with special focus on recognizing seizures and other abnormal EEG activity profiles.

iii) Dr. Hao learned coding in Matlab and animal applied her knowledge by coding the EEG power spectrum analysis and an interface to simultaneous display of EEG, Ca imaging and mouse activity. (Figure 4).

iv) Dr. Hao completed all animal welfare and animal handling trainings provided by Baylor College of Medicine and learned how to handle mice and their breeding by managing the mouse colonies.

Results Disseminated to communities of interest:

Nothing to Report yet.

Plans for the next reporting period:

We plan to continue the experiments and meet the goals as stated in the SOW. We expect that there will be a delay with respect to meeting those goals, commensurate with the delay that it will take for the Award to transfer between institutions. Currently experiments were suspended on 6/1/2016 pending this transfer. We hope that by 11/1/2016 we will be able to resume experiments. To work towards this goal, we have already obtained IACUC permission to perform experiments in the new institution (Jamaica Plain VA Campus of the Boston Veterans Administration System). In parallel Dr Hao has obtained a new H1 visa giving her permission to work in the new institution.

Assuming a restart date of 11/1/2016, we anticipate to complete our fist yearly report on 3/1/2016. At that time we will report on the first 12 months of the project goals as ourlined in the SOW. We expect that at that time we will have :

1) Optimize the identification of abnormal EEG patterns in collaboration with Drs Noebels and Maheshwari,
2) Optimize the TeT delivery to maximize animal survival and development of focal epilepsy

3) Obtained 2-photon recordings from the pyramidal neurons of an initial cohort of ~5 animals with TeT focal epilepsy
4) Initiate 2-photon experiments to record from the PV+ interneurons of TeT injected animals

4. IMPACT:

We are still at the initial stage of the project. There have been no publications yet, in order to have impact.

The impact of our project on:
<u>1) the development of the principle discipline:</u> nothing to report
<u>2) the disciplines:</u> nothing to report
<u>3) technology transfer:</u> nothing to report
<u>4) society beyond science and technology:</u> nothing to report

5. CHANGES / PROBLEMS

The PI's laboratory is moving from Baylor College of Medicine to Brigham and Women's Hospital and the Jamaica Plain Hospital, Harvard Medical School. The DoD experiments will be performed at the Jamaica Plain Veterans Administration Hospital location and will be administered by the Boston VA Research Institute, Inc. (BVARI) at Jamaica Plain VA, 150 S Huntington Ave, Boston, MA.

This transfer is a positive development, as additional resources pertaining to the execution of this project, and to the study of epilepsy in general, will now be available to the PI. For example, the PIs 2-photon setup will be upgraded to have the capability of spatial light modulation stimulation technology. This will allow the PI to activate arbitrarily chosen neuronal units, which genetically express C1V1 channel rhodopsin, thereby causally testing the hypotheses that will be developed by the execution of the current project. However, transferring the laboratory inevitably will cause a brief interruption of the experimental process, that was not accounted for in the current SOW.

An added complication has been that Dr Hao, who is the primary postdoctoral fellow experimenter of the project, lost her legal status in the US during the transfer process. Dr. Hao was working under a student F1 OPT visa at Baylor College of Medicine, waiting for the approval of the H1b petition that Baylor had submitted for her. Baylor International Student Office decided to retract the H1b petition because Dr. Hao was moving to Boston. The retraction letter was sent without any communication with the USCIS and at the very moment Dr. Hao's H1b petition was approved. Dr. Hao thus lost both her F1 status (because of the H1b approval) and her H1b status (because of the retraction letter, see appendix). USCIS denied the request to resume Dr. Hao's F1 status. As a consequence, she had to leave the country in May 2016 at short notice. She could not come back to US before Boston VA finished the hire process, filing another H1b petition. Her H1b petition was approved on Jun 16^{th}. She received her passport with H1b stamp on July 30^{th}. She arrived in Boston on August 22^{nd} and is

currently completing the required procedure for being added to the already approved JP VA IACUC protocol for the DoD experiments.

Overall, we expect that the transfer of the grant will incur a delay of approximately 5 months, i.e. from 6/1/2016 (when the grant was relinquished from Baylor) to ~11/1/2016 (when we estimate the transfer process will hopefully be completed). We therefore expect that our goals as presented in the SOW will be delayed by 5 months.

We note that there has been no change in approach and that, despite the delay, we still plan to meet all stated goals of the project, at no extra cost. =

6. PRODUCTS

Nothing to report so far.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name: Stelios Smirnakis
Project Role: Principal Investigator
Researcher Identifier: orcid.org/0000-0002-1929-2811
Nearest person month worked: 1.6 months
Contribution to Project: Conceive and Design the project. Participate in experimental planning and analysis.

Name: Jochen Meyer
Project Role: Instructor
Researcher Identifier: orcid.org/0000-0002-3976-3334
Nearest person month worked: 3
Contribution to Project: Participate in training, experiments and analysis.

Name: Zhaozhe Hao
Project Role: Postdoctoral Associate
Researcher Identifier: no orcid id yet.
Nearest person month worked: 7 (1 month lost due to the Visa issue)
Contribution to Project: conduct the experiment, data analysis

Name: Jeff Noebels
Project Role: Consultant/Collaborator
Researcher Identifier: orcid.org/0000-0002-2887-0839
Nearest person month worked: 0.1 calendar months
Contribution to Project: Provide advice, and help with EEG recordings.

This is the first reporting period. No other organizations were involved as partners. Following the PIs transfer, the main organization where research will be performed will be the Jamaica Plain Veterans Administration Research Campus. At that time, Dr Noebels and Meyer at Baylor College of Medicine will be funded through subcontracts.

8. SPECIAL REPORTING REQUIREMENTS:

None.

9. APPENDIX

The loss-of-status notice sent by Emilie Gorden, the International Student Service Officer of Dr. Hao's F1 status sponsor institution (Baylor College of Medicine). Dr Hao is now back to the US as an employee of BVARI at the Jamaic Plain Veterans Administration Hospital.

>>>> From: Gordon, Emilie <emilie.gordon@ou.edu>
>>>> Sent: Monday, April 25, 2016 1:29 PM
>>>> To: Hao, Zhaozhe
>>>> Subject: RE: I 20 for opt extension
>>>>
>>>> Hello Zhaozhe,
>>>>
>>>> Unfortunately I do not have good news. After much back and forth between OU, Baylor, the USCIS, and SEVP, it is my unpleasant task to announce to you that our correction request was not approved by SEVP. Although we were able to produce a detailed letter of the facts, your H1B approval notice, the withdrawal notice from the USCIS sent in April, and the letter Baylor sent to withdraw your H1B application, SEVP would not correct your status without proof that the USCIS received the withdrawal letter before your H1B was approved, which the USCIS could not produce.
>>>>
>>>> Therefore, you remain in terminated F-1 status, and your H1B has been revoked. Unfortunately at this point, not only must you cease all employment, but you must also leave the country. It is my hope that your new institution will consider applying for an H1B for you so you may get the visa while abroad in order to come back to the US. If they will not sponsor your H1, they may consider a J1 visa. I recommend you speak with them to find out. Please let me know if there is anything else I can do for you. I am sorry we were not able to reactivate your F1 status.
>>>>
>>>> Sincerely,
>>>>
>>>> Emilie Gordon
>>>> Int'l Student Advisor|SEVIS Analyst International Student Services
>>>> College of International Studies
>>>> 729 Elm Avenue, 144, Norman, OK 73019-2102 ou.edu/international |
>>>> 405.325.3337 | iss@ou.edu like ISS: FACEBOOK | follow CIS: TWITTER